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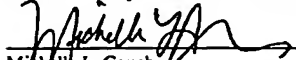


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Date: October 23, 2003

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This is a request for a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

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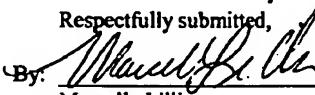
Customer No. 27476		Docket No. 21455.001	Type a plus sign (+) inside this box → +
<b>INVENTOR(S)/APPLICANT(S)</b>			
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Magagnoli	Claudia		
<b>TITLE OF INVENTION (280 characters max)</b>			
METHOD OF PURIFYING LTK63			
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STATE: California	ZIP CODE: 94662-8097	COUNTRY: USA	
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<input type="checkbox"/> Other (specify)			
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States government.

☒ No

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# METHOD OF PURIFYING LTK63

## INTRODUCTION

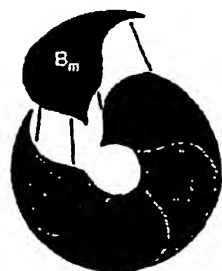
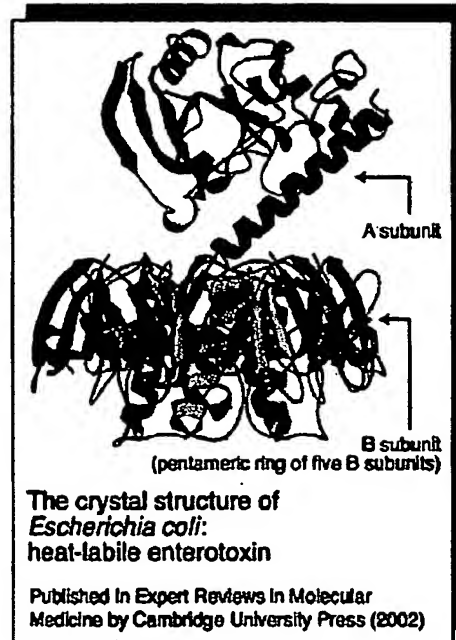


to identify an analytical method able to give information on integrity of the LT K63 molecule, produced and purified in the Technology Development Dept., to be utilized as mucosal adjuvant.

K63, an oligomeric protein of about 82 KDa, is a non-toxic mutant of LT (heat-labile enterotoxin), obtained by site-specific mutagenesis on subunit A, that retains the structural organization of the native molecule.

LT K63 subunit A is composed by a single polypeptidic chain of 240 aminoacids, with a MW of 27 KDa; subunit B is a pentamer formed by 5 identical monomers of 103 aa each, with a MW of 55 KDa. Both subunits contain high percent of positive charged aa; (sub. A IP = 6,3; sub. B<sub>5</sub> IP =9,1; AB<sub>5</sub> IP = 8,5).

[conventionally, the entire protein is indicated as AB<sub>5</sub>, the separate subunits as A and B<sub>5</sub>, and the single monomer of the B subunit as B<sub>m</sub>].



Analytical techniques currently used to characterize the LT K63 protein (electrophoresis and immunoblotting, mass spectrometry, aminoacid analysis) do not allow to monitor the AB<sub>5</sub> form of the molecule in comparison to A or B<sub>5</sub>: for example, in SDS-PAGE the protein is visible as separate subunit, A and B<sub>5</sub> in not denaturing or A and B<sub>m</sub> in denaturing conditions.

GF-HPLC is the single method able to observe the AB<sub>5</sub> form of the protein in comparison to A or B<sub>5</sub>.

Unfortunately, Gel Filtration columns in use till now did not permit a good separation of the AB<sub>5</sub> and B<sub>5</sub> peaks, because of their extremely close retention times.

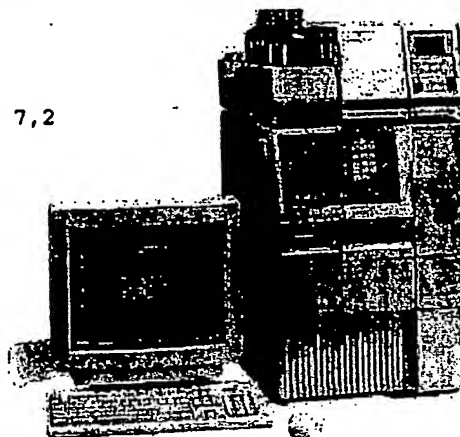
On the left: schematic representation of AB<sub>5</sub> (top) and B<sub>5</sub> (bottom) forms of LT K63

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## "Old" GF-HPLC analysis on TSK G3000SWxl

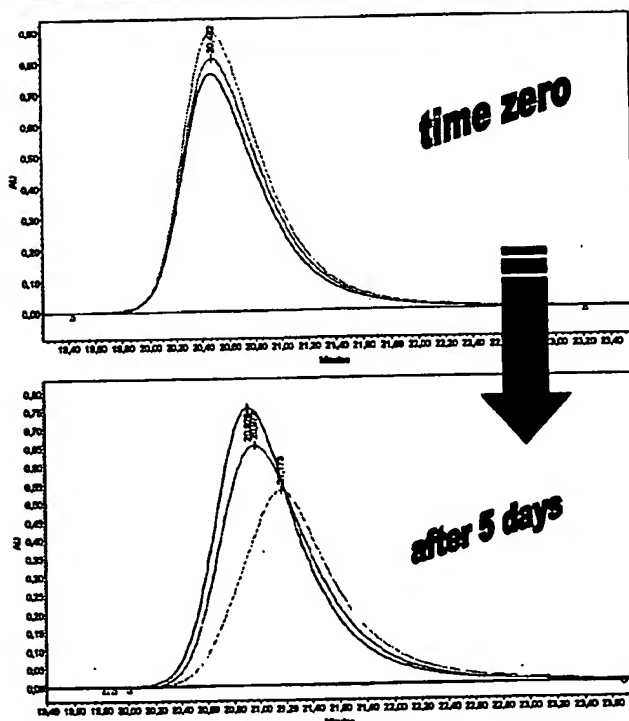
Instrument: Alliance 2695 Waters  
 Buffer: KPi 100 mM + Na<sub>2</sub>SO<sub>4</sub> 100 mM pH 7,2  
 Flow: 0,5 ml/min  
 Detection: PDA 996 @ 214 and 280 nm

Column: TSK G3000SWxl Tosoh  
 Material: silica gel  
 Surface mod: residual -OH groups  
 Particle size: 5 µm  
 Porosity: 250 Å



Difference in RT (Retention Times) of the AB<sub>5</sub> and B<sub>5</sub> forms is minimum and the peaks are not well resolved, with a difficult determination of B<sub>5</sub> content in K63 samples; B<sub>5</sub> less than 20% is not detectable.

Sample Name	Injection Volume	Channel	Retention
K63 in PBS	100,00	214nm	4,00
K63 in Chaps 0,25%	100,00	214nm	4,00
K63 in citrate	100,00	214nm	4,00



K63 purified samples in which content of B<sub>5</sub> respect to AB<sub>5</sub> varies during storage, valued by SDS-PAGE (not shown) and GF-HPLC:

at time zero samples presented similar electrophoretic patterns, and superimposition of the respective chromatograms showed RT and peak profiles almost identical.

After five days, SDS-PAGE shows that in one sample the percent of B<sub>5</sub> has increased; in GF-HPLC the corresponding peak (green) is tailed and its RT moves to the right.

However, this separation does permit only a qualitative valuation of degradation of the AB<sub>5</sub> molecule.

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# "Novel" GF-HPLC analysis on Ultrahydrogel 250

after 5 days

Column: Ultrahydrogel 250 Waters  
Material: hydroxylated polymetacrylate  
Surface mod: residual -COOH groups  
Particle size: 6 µm  
Porosity: 250 Å

Other conditions: same as before

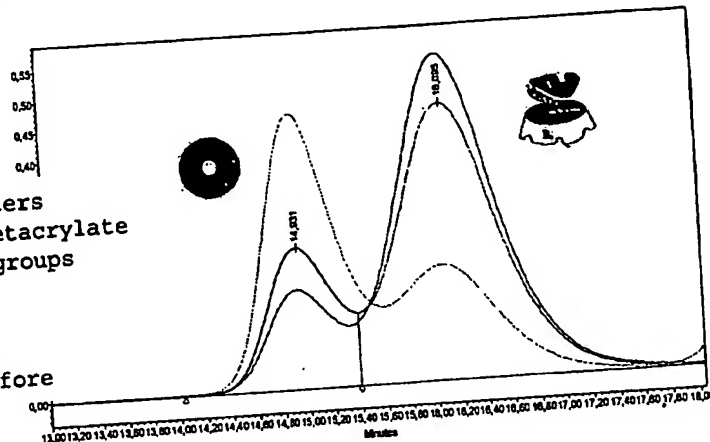
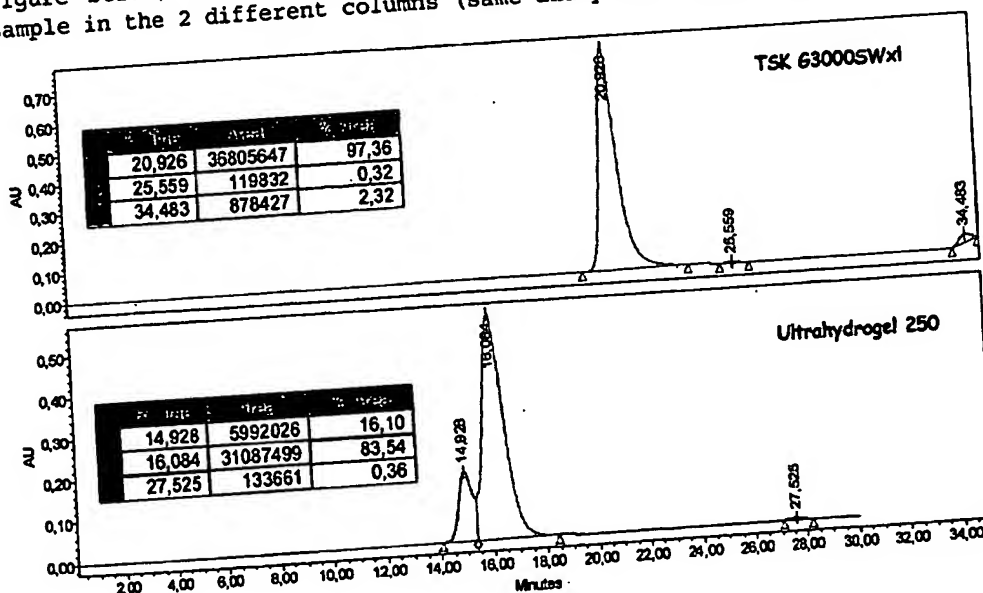


Figure above shows the analysis of the same samples repeated on Ultrahydrogel 250 column.

## 2 peaks instead one!

By comparison with TSK G3000SWx1 and with SDS-PAGE data became natural to attribute the peak with smaller RT to B<sub>5</sub>; this suggests that the separation mechanism is not purely Gel Filtration, or that the relative dimensions of the molecules are not proportional to their MW.

Figure below, compare values of Area and Area % obtained for the same K63 sample in the 2 different columns (same analytical conditions).



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## Optimizing elution conditions

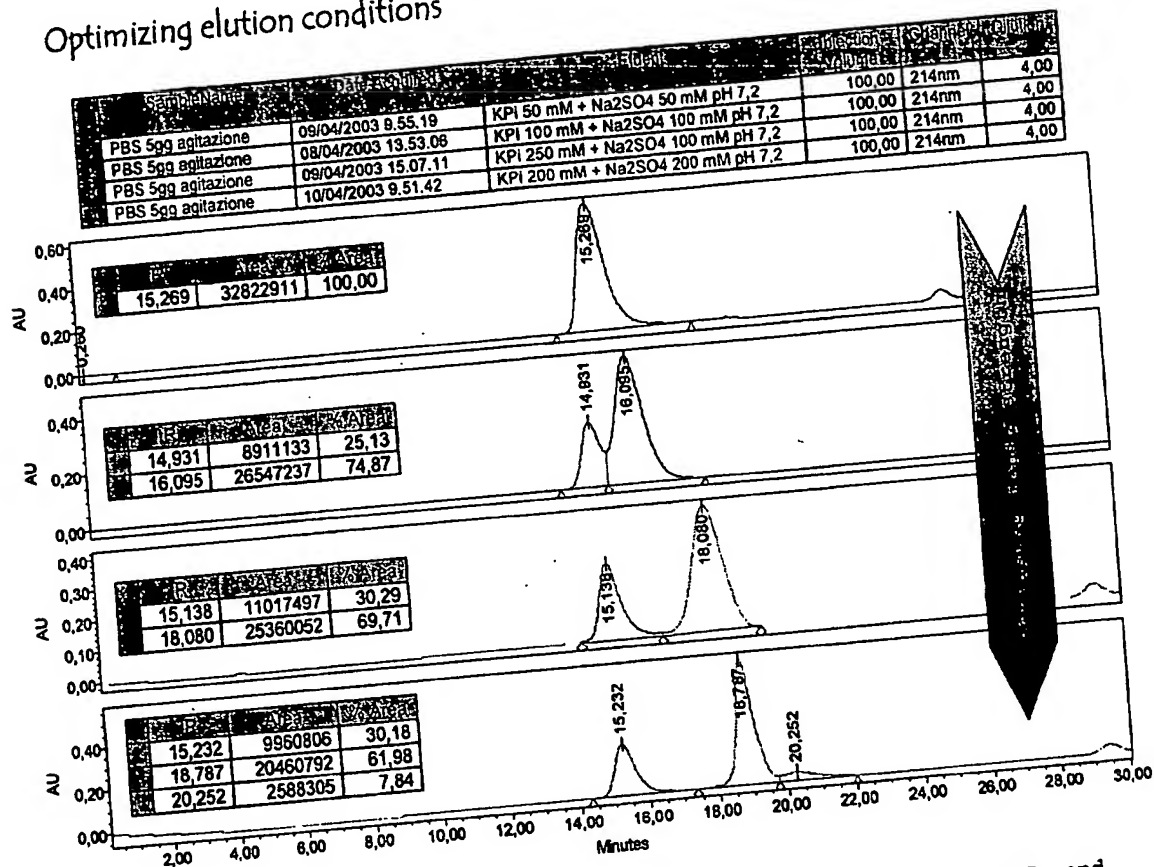
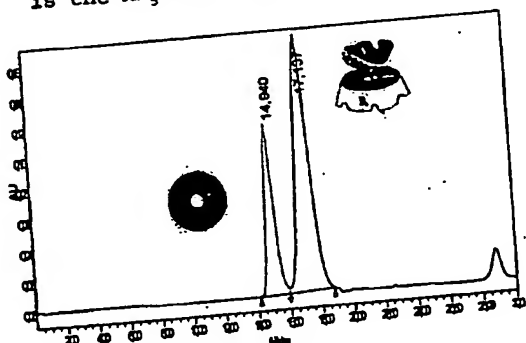


Figure above shows the effect of ionic strength on selectivity of AB<sub>5</sub> and B<sub>5</sub> peaks in Ultrahydrogel column. Higher ionic strength causes a more net separation of the 2 peaks, until partial degradation of AB<sub>5</sub> when saline concentration reaches 200 mM.

Curiously, peak that mostly results affected by ionic strength variation is the AB<sub>5</sub> one, while RT of B<sub>5</sub> peak (in red) remains almost equal.



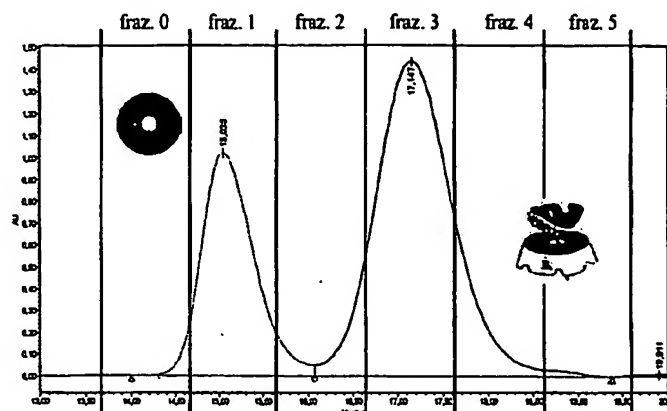
On the left, the chromatographic profile of LT K63 sample in the elution buffer chosen:

KPI 200 mM + Na<sub>2</sub>SO<sub>4</sub> 100 mM pH 7,2

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## Attributing AB<sub>5</sub> and B<sub>5</sub> peaks: Fractionation and SDS-PAGE

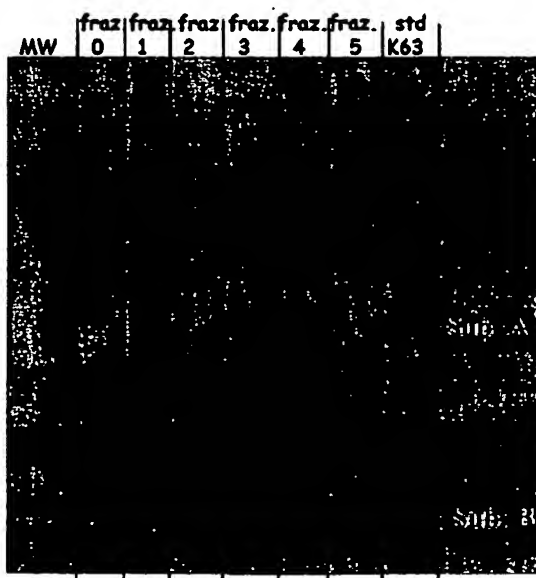
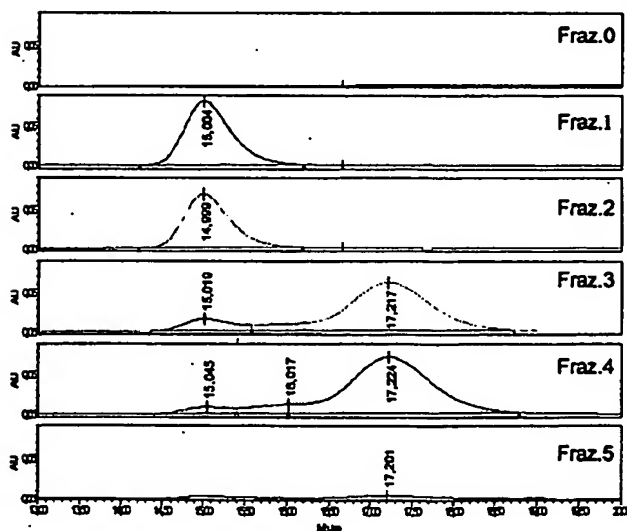
To verify the attribution of peaks obtained in GF-HPLC to the AB<sub>5</sub> and B<sub>5</sub> forms of the protein, a K63 sample was **fractionated** for further investigation: it was injected 3 times and 6 fractions of 500 µl volume were collected for each run since 13,8 to 19,8 minutes. Same fractions of the single runs were then pooled to obtain a final volume of 1,5 ml/fraction.



214 nm chromatogram of a single fractionating run

Fraction 0-5 were then re-injected in the HPLC system (bottom left) and analyzed by SDS-PAGE (bottom right), and they confirm what previously supposed: **peak with lower RT, present in fractions 1 and 2, contains only B<sub>5</sub>** (visible in SDS-PAGE as monomer B<sub>m</sub>), while **peak with higher RT, present in fractions 3 and 4 migrates in SDS-PAGE as 2 distinct bands of A and B<sub>m</sub>**.

214 nm chromatograms of the re-injected fractions

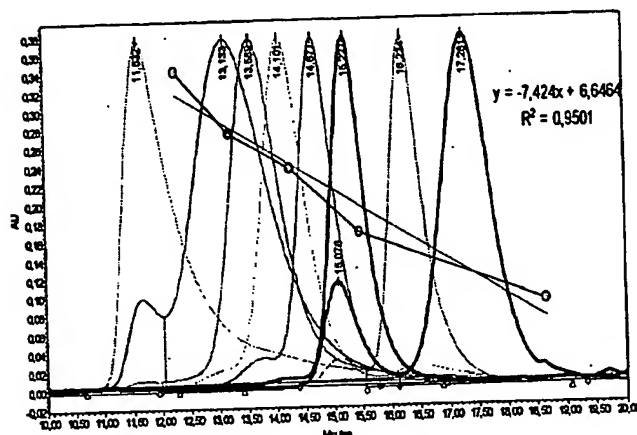


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## Dimensional characterization: Apparent Molecular Weight

A calibration curve of the Ultrahydrogel column was made with standard proteins of known MW. The corresponding  $R^2$  was 0,95.

Peak's retention time of CRM<sub>197</sub> ref. protein on the curve gave an apparent MW of 57 KDa (56,9 theoretical); B<sub>5</sub> apparent MW on the same curve resulted 65 KDa (55 theor.). AB<sub>5</sub> MW resulted 9,6 KDa (82 theor.), confirming that separation mechanisms other than Gel Filtration act in this case.



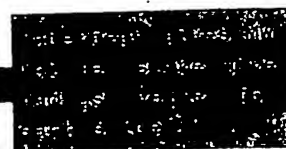
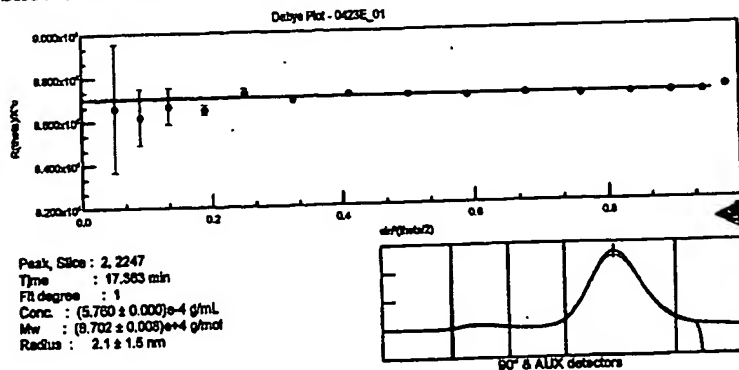
Standard proteins	Rt (min)	M <sub>w</sub> (Da)
Thyroglobulin (bovine)	11:62	669.000
Apoferitin	13:13	476.316
B-amylase	13:58	224.340
Alcohol Deydrogenase	14:10	146.980
BSA	14:57	66.800
Carbonic Anhydrase	16:22	29.023
Sample proteins	Rt (min)	M <sub>w</sub> exp.
CRM	15:23	57.099
K63 AB <sub>5</sub>	17:26	9.611
K63 B <sub>5</sub>	15:07	65.607

Superimposition of standard proteins, CRM<sub>197</sub> reference (bold blue), K63 (bold red) and calibration curve used for apparent MW determination.

## Dimensional characterization: Light Scattering analysis

Further characterization on GF-HPLC peaks was obtained by use of on-line Light Scattering (MALLS) detector coupled to GF-HPLC: 18 angle Dawn EOS Wyatt.

the Debye plot relative to LS analysis of AB<sub>5</sub> peak is shown below



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## Dimensional characterization: Light Scattering analysis

Table below groups MALLS data for 3 different K63 samples and for BSA used as control of instrument good functioning.

Following parameters are indicated:

- ✓ Absolute MW in Daltons (not referred to calibration curves)
- ✓ Peak poly-dispersion (value of 1 for mono-dispersed molecules = proteins)
- ✓ gyration radius in nm (measure of molecular dimension; sens. lower limit = 10nm)

(percent next to each value indicates instrumental variability)

sample	peak	MW <sub>theor</sub>	MW <sub>exp</sub>	%	Polydisp. Mw/Mn	%	Rz	%
BSA	monomer	66.800	65.970	0,3	1,000	0,5	6,5	5

sample	peak	MW <sub>theor</sub>	MW <sub>exp</sub>	%	Polydisp. Mw/Mn	%	Rz	%
K63 in 20 mM phosphate	AB <sub>5</sub>	82.000	85.450	0,3	1,001	0,4	5,1	5
K63 in 0,05% chaps	AB <sub>5</sub>	82.000	85.300	0,3	1,001	0,5	6,5	6
K63 in 0,25% chaps	AB <sub>5</sub>	82.000	85.470	0,3	1,000	0,4	4,3	5

sample	peak	MW <sub>theor</sub>	MW <sub>exp</sub>	%	Polydisp. Mw/Mn	%	Rz	%
K63 in 20 mM phosphate	B <sub>5</sub>	55.000	58.030	0,4	1,000	0,6	16,5	3
K63 in 0,05% chaps	B <sub>5</sub>	55.000	57.030	0,4	1,000	0,6	15,2	5
K63 in 0,25% chaps	B <sub>5</sub>	55.000	57.530	0,5	1,000	0,6	19,9	5

### Gyration Radius

In all the samples, peak with the higher RT (AB<sub>5</sub>) shows smaller value of gyration radius in comparison with B<sub>5</sub>. A possible explanation of the unusual chromatographic behaviour of AB<sub>5</sub> molecule is that, despite its heavier MW, its conformation results more compact than B<sub>5</sub> alone.

[this supposition has to be confirmed with other techniques, because MALLS values are close to the sensitivity lower limit and show high variability]

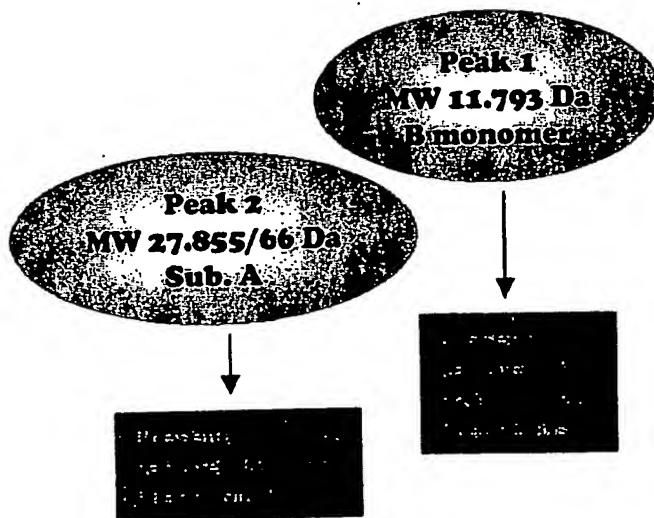
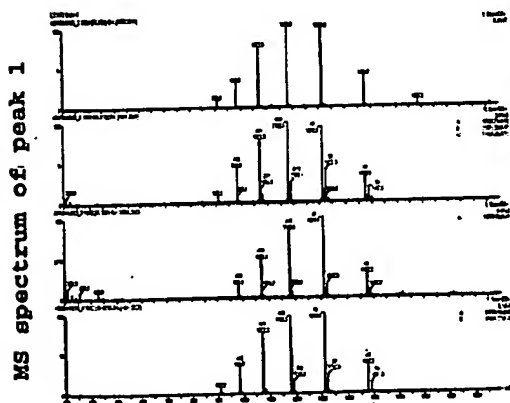
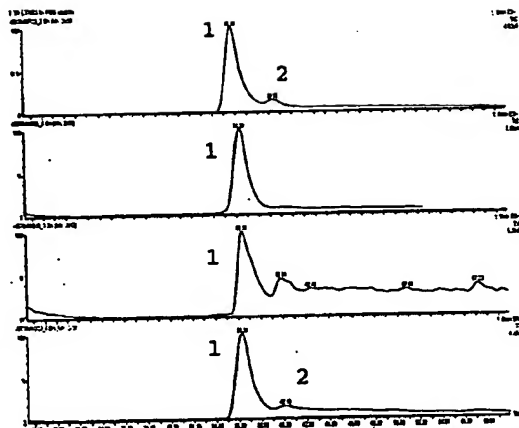
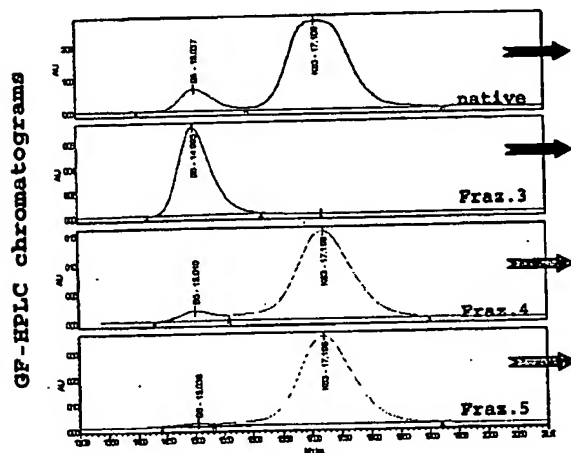
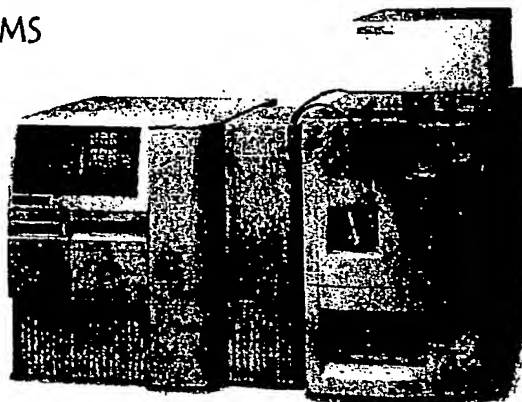
### Molecular Weight

Results are quite similar for all the K63 samples; 2 mono-dispersed peaks are present, with a MW of about 57 and 85 KDa in accord with the expected values for B<sub>5</sub> and AB<sub>5</sub>. [N.B. the dn/dc ratio used was not determined experimentally, and this can explain at least in part the discrepancy between theoretical and experimental data]

## Dimensional characterization: LC - ESI- MS

Instrument: Alliance 2695 Waters  
 Detection: PDA 996 Waters  
 MS ZQ 4000 Micromass  
 RP column: Jupiter Phenomenex C4  
 300 Å

A native K63 + 3 samples coming by  
 GF-HPLC fractionation were  
 analysed on LC-ESI-MS to confirm  
 peak attribution:



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# conclusions

- a GF-HPLC method able to discriminate AB<sub>5</sub> from B<sub>5</sub> was set up: this improves our capability to study degradation process of the protein and possibility of its stabilization.
- elution conditions were optimised: effect of ionic strength of eluent buffer was investigated, and it appears to influence especially the retention time of AB<sub>5</sub>.
- peaks attribution was verified: fractioning and SDS-PAGE permitted to identify B<sub>5</sub> and AB<sub>5</sub> peaks.
- dimensional analysis: apparent Molecular Weight determination indicates that other separation mechanism than Gel Filtration acts at least for AB<sub>5</sub>.
- dimensional analysis: MALLS absolute MW result in accord with theoretical values for AB<sub>5</sub> and B<sub>5</sub> subunit; dispersity indicates that peaks are composed of homogeneous material; dimensional values suggest that AB<sub>5</sub> is in a more compact conformation respect to B<sub>5</sub>.
- dimensional analysis: LC-ESI-MS data provide another proof of peak attribution.

## future works ....

- hydrodynamic radius determination
  - GF-HPLC elution at different pH
  - experimental dn/dc determination
-

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